PROJECT NUMBER: 1904
PROJECT TITLE: Tobacco Physiology and Biochemistry
PROJECT LEADER: H. Y. Nakatani
PERIOD COVERED: November, 1988

I. LOW NICOTINE STUDY

A. **Objective**: To investigate the biochemistry of the nicotine biosynthetic pathway at putrescine N-methyltransferase (PMT) and specifically to isolate PMT from tobacco root extracts.

B. **Status**: The roots from the group 15 tobacco plants were harvested and stored at -80°C. The roots from group 14 and the remaining roots from group 13 have been processed to the ammonium sulfate (40-65%) stage. In addition two extracts (ammonium sulfate) have been prepared from the group 15 harvest (1). Bulk ammonium sulfate samples have been processed through the phenyl-Sepharose column (2).

Additional studies have been conducted with the S-adenosyl-homocysteine (SAH) affinity matrix for the purification of PMT. It appears that PMT may bind to this matrix by two modes of interactions a) affinity and b) ion-exchange or other mechanism(s) (2). PMT and other methyltransferases which are bound in the affinity mode are eluted from this column with 1 mM SAM; PMT and other proteins bound in the latter mode(s) are then removed by elution with 0.5 M NaCl (2). An SAH-column which was blocked with ethanolamine rather than mercaptoethanol was not found to function better than the mercaptoethanol blocked matrix (5,6). Additional studies were also conducted with the ω-aminoethyl agarose (AEA) matrix. This latter column was found to be promising for the purification of PMT (5,6). A SAM-eluted PMT fraction from the SAH column was applied to the AEA column. The peak fraction having PMT-activity upon elution with 5 mM putrescine showed a specific activity of 51,000 units (nmoles/mg protein at 30°C for 30 min.) (5,6). Two preparations of PM extracts (PM 84 and 85) from the ammonium sulfate stage were processed through the following series: phenyl-Sepharose, SAH-affinity and AEA-affinity columns. The pooled samples after the AEA-affinity column in both cases showed an increase in specific activity of about 500-fold compared with the ammonium sulfate stage. Moreover, the specific activities of the pooled PMT-active fractions were >11,000 (1,2,3,5,6). A set of samples from the various purification steps from PM 84 were prepared and sent to PDI (Protein Database Incorporated) for 2-D examination (3). SDS-Page examination from these samples were also obtained (3). A sample from the SAH stage was submitted for ^{3}H-SAM photoaffinity labeling (Dr. Bruce Davies).

Further studies were continued with the SAH column to obtain greater amounts of PMT for processing through the AEA column. Following elution of PMT-activity with SAM, the column was washed with dialysis buffer. PMT-activity was again eluted with SAM after applying additional PMT-active samples (phenyl-Sepharose stage) to this column (5,6). This elution scheme decreases the processing

time to obtain PMT from the SAH column without having to recycle the SAH matrix completely.

A number of miscellaneous studies have also been conducted. Examination of the N-methylputrescine affinity (NMP) matrix was continued. However, no increase in specific activity of PMT over the starting material was obtained for any of the NMP-affinity matrices tested. These results precluded its use at this time although recovery of PMT activity ranged from 6-25% of the applied sample (from prep 1) (4). A longer separating gel was examined in the ELFE system in an attempt to increase the resolution of the native gel system. This was not helpful since the increased electrophoresis time resulted in the loss of PMT activity (3). The Rotofor is being examined as a possible means of resolving the most purified samples by isoelectric focusing. 1 mM SAM was not found to increase the stability of PMT preparations over a three day time course at 4°C (3). Dr. Walter P. Hempfling has also reconfirmed that the molecular weight of PMT is ~60,000 daltons by using a P100 BioGel matrix (Bio-Rad) gel filtration column. This is consistent with previous results obtained with the Pharmacia Sephacryl gel filtration columns (4). More sensitive methods for assaying proteins are being investigated: a fluorescence method using fluorescamine and a Protein-Gold reagent (Integrated Separations System) (4).

C. **Plans**: Continue to process roots to the 40-65% ammonium sulfate stage. Prepare enriched PMT samples from the ammonium sulfate stage using phenyl-Sepharose and SAH and AEA-columns. Determine efficacy of one of the more sensitive protein assay methods for use in our studies. Evaluate other classical chromatographic methods to yield a homogeneous PMT preparation.

D. **References**:

1. Dunn, R. L. Notebook No. 7899.
2. Malik, V. Notebook No. 8542.
3. Davies, S. Notebook No. 8694.
4. Yu, T. Notebook No. 8381.
5. Mooz, E. D. Notebook No. 8599.
6. Crockett, E. Notebook No. 8563.